ICAM-4 MATERIALS AND METHODS

This application is a continuation-in-part of U.S. Patent Application Serial No. 08/656,984, filed June 6, 1996 and currently pending, is a continuation-in-part of U.S. Patent Application Serial No. 08/481,130, filed June 7, 1995 and currently pending, which is a continuation-in-part of U.S. Patent Application Serial No. 08/245,295, filed May 18, 1994 and currently pending, which in turn is a continuation-in-part of U.S. Patent Application Serial No. 08/102,852, filed August 5, 1993 and now abandoned, which is a continuation-in-part of U.S. Patent Application Serial No. 08/009,266, filed January 22, 1993 and now abandoned, which is a continuation-in-part of U.S. Patent Application Serial No. 07/894,061, filed June 5, 1992 and now abandoned, which is a continuation-in-part of U.S. Patent Application Serial No. 07/889,724, filed May 26, 1992 and now abandoned which is a continuation-in-part of co-pending U.S. Patent Application Serial No. 07/827,689, filed January 27, 1992 and now abandoned.

FIELD OF THE INVENTION

The present invention relates generally to cellular adhesion molecules and more particularly to the cloning and expression of DNA encoding a heretofore unknown polypeptide designated "ICAM-4" which possesses structural relatedness to the intercellular adhesion molecules ICAM-1, ICAM-2, and ICAM-R.

BACKGROUND OF THE INVENTION

Research spanning the last decade has significantly elucidated the molecular events attending cell-cell interactions in the body, especially those events involved in the movement and activation of cells in the immune system, and more recently, those involved in development and normal physiological function of cells in the nervous system. See generally, Springer, *Nature, 346*: 425-434 (1990) regarding cells of the immune system, and

Yoshihara, *et al.* *Neurosci.Res.* *10*:83-105 (1991) and Sonderegger and Rathjen, *J.Cell Biol.* *119*:1387-1394 (1992) regarding cells of the nervous system. Cell surface proteins, and especially the so-called Cellular Adhesion Molecules ("CAMs") have correspondingly been the subject of pharmaceutical

5   research and development having as its goal intervention in the processes of leukocyte extravasation to sites of inflammation and leukocyte movement to distinct target tissues, as well as neuronal differentiation and formation of complex neuronal circuitry. The isolation and characterization of cellular adhesion molecules, the cloning and expression of DNA sequences encoding

10   such molecules, and the development of therapeutic and diagnostic agents relevant to inflammatory processes and development and function of the nervous system have also been the subject of numerous U.S. and foreign applications for Letters Patent. See Edwards, *Current Opinion in Therapeutic Patents, 1(11):* 1617-1630 (1991) and particularly the published "patent

15   literature references" cited therein.

Of fundamental interest to the background of the present invention are the prior identification and characterization of certain mediators of cell adhesion events, the "leukointegrins," LFA-1, MAC-1 and gp 150.95 (referred to in WHO nomenclature as CD18/CD11a, CD18/CD11b, and

20   CD18/CD11c, respectively) which form a subfamily of heterodimeric "integrin" cell surface proteins present on B lymphocytes, T lymphocytes, monocytes and granulocytes. See, e.g., Table 1 of Springer, *supra,* at page 429. Also of interest are other single chain adhesion molecules (CAMs) that have been implicated in leukocyte activation, adhesion, motility and the like,

25   which are events attendant to the inflammatory process. For example, it is presently believed that prior to the leukocyte extravasation which characterizes inflammatory processes, activation of integrins constitutively expressed on leukocytes occurs and is followed by a tight ligand/receptor interaction between the integrins (e.g., LFA-1) and one or both of two distinct intercellu-

30   lar adhesion molecules (ICAMs) designated ICAM-1 and ICAM-2 which are

expressed on blood vessel endothelial cell surfaces and on other leukocytes.

    Like the other CAMs characterized to date, [e.g., vascular adhesion molecule (VCAM-1) as described in PCT WO 90/13300 published November 15, 1990; and platelet endothelial cell adhesion molecule (PECAM-1) as described in Newman *et al., Science, 247*: 1219-1222 (1990) and PCT WO 91/10683 published July 25, 1991], ICAM-1 and ICAM-2 are structurally homologous to other members of the immunoglobulin gene superfamily in that the extracellular portion of each is comprised of a series of domains sharing a similar carboxy terminal motif. A "typical" immunoglobulin-like domain contains a loop structure usually anchored by a disulfide bond between two cysteines at the extremity of each loop. ICAM-1 includes five immunoglobu- lin-like domains; ICAM-2, which differs from ICAM-1 in terms of cell distribution, includes two such domains; PECAM-1 includes six; VCAM includes six or seven, depending on splice variations, and so on. Moreover, CAMs typically include a hydrophobic "transmembrane" region believed to participate in orientation of the molecule at the cell surface and a carboxy terminal "cytoplasmic" region. Graphic models of the operative disposition of CAMs generally show the molecule anchored in the cell membrane at the transmembrane region with the cytoplasmic "tail" extending into the cell cytoplasm and one or more immunoglobulin-like loops extending outward from the cell surface.

    A number of neuronal cells express surface receptors with extracellular Ig-like domains, structurally similarity to the ICAMs. See for example, Yoshihara, *et al., supra*. In addition to Ig-like domains, many adhesion molecules of the nervous system also contain tandemly repeated fibronectin-like sequences in the extracellular domain.

    A variety of therapeutic uses has been projected for intercellular adhesion molecules, including uses premised on the ability of ICAM-1 to bind human rhinovirus. European Patent Application 468 257 A published January 29, 1992, for example, addresses the development of multimeric configura-

tions and forms of ICAM-1 (including full length and truncated molecular forms) proposed to have enhanced ligand/receptor binding activity, especially in binding to viruses, lymphocyte associated antigens and pathogens such as *Plasmodium falciparum*.

5   In a like manner, a variety of uses has been projected for proteins immunologically related to intercellular adhesion molecules. WO91/16928, published November 14, 1991, for example, addresses humanized chimeric anti-ICAM-1 antibodies and their use in treatment of specific and non-specific inflammation, viral infection and asthma.   Anti-

10   ICAM-1 antibodies and fragments thereof are described as useful in treatment of endotoxic shock in WO92/04034, published March 19, 1992. Inhibition of ICAM-1 dependent inflammatory responses with anti-ICAM-1 anti-idiotypic antibodies and antibody fragments is addressed in WO92/06119, published April 16, 1992.

15   Despite the fundamental insights into cell adhesion phenomena which have been gained by the identification and characterization of intercellular adhesion proteins such as ICAM-1 and lymphocyte interactive integrins such as LFA-1, the picture is far from complete. It is generally believed that numerous other proteins are involved in inflammatory processes

20   and in targeted lymphocyte movement throughout the body. For example, U.S. Patent Application Serial Nos. 07/827,689, 07/889,724, 07/894,061 and 08/009,266 and corresponding published PCT Application WO 93/14776 (published August 5, 1993) disclose the cloning and expression of an ICAM-Related protein, ICAM-R.   The disclosures of these applications are

25   specifically incorporated by reference herein and the DNA and amino acid sequences of ICAM-R are set out in SEQ ID NO. 4 herein. This new ligand has been found to be expressed on human lymphocytes, monocytes and granulocytes.

          Of particular interest to the present application, still another

30   ICAM-like surface molecule was identified which has a tissue specific

expression unlike that of any known ICAM molecule. Mori, *et al.*, [*Proc.Natl.Acad.Sci.(USA) 84*:3921-3925 (1987)] reported identification of a telencephalon-specific antigen in rabbit brain, specifically immunoreactive with monoclonal antibody 271A6. This surface antigen was named

5   telencephalin. Imamura, *et al.*, [*Neurosci.Letts. 119*:118-121 (1990)], using a polyclonal antibody to assess localized expression, asserted that expression of telencephalin in visual cortex of cats showed variation in layers of the tissue, and also reported telencephalin expression was variable as a function of development. Oka, *et al.*, [*Neuroscience 35*:93-103 (1990)] subsequently

10   reported isolation of telencephalin using monoclonal antibody 271A6. The publication reports a molecular weight for the surface molecule of about 500 kD and that the molecule was composed of four subunits, each with a native molecular weight of 130 kD and approximately 100 kD following *N*-glycanase treatment. Yoshihiro, *et al.*, [*Neuroscience*, Research Supplement 18, p. S83

15   (1994)], reported the cDNA and amino acid sequences for rabbit telencephalin at the 17th Annual Meeting of the Japan Neuroscience Society in Nagoya, Japan, December 7-9, 1993, and the 23rd Annual Meeting of the Society for Neuroscience in Washington, D.C., November 9, 1993 [Society for Neuroscience Abstracts 19 (1-3) p. 646 (1993)]. The deduced amino acid

20   sequence reported suggested that the 130 kD telencephalon is an integral membrane protein with nine extracellular immunoglobulin (Ig)-like domains. The distal eight of these domains showed homology to other ICAM Ig-like domains. This same information was reported by Yoshihara, *et al.*, in *Neuron 12*:543-553 (1994).

25   There thus continues to be a need in the art for the discovery of additional proteins participating in human cell-cell interactions and especially a need for information serving to specifically identify and characterize such proteins in terms of their amino acid sequence. Moreover, to the extent that such molecules might form the basis for the development of

30   therapeutic and diagnostic agents, it is essential that the DNA encoding them

be elucidated. Such seminal information would *inter alia*, provide for the large scale production of the proteins, allow for the identification of cells naturally producing them, and permit the preparation of antibody substances or other novel binding proteins specifically reactive therewith and/or inhibitory of ligand/receptor binding reactions in which they are involved.

BRIEF SUMMARY OF THE INVENTION

In one of its aspects, the present invention provides purified and isolated polynucleotides (e.g., DNA sequences, RNA transcripts and anti-sense oligonucleotides thereof) encoding a novel polypeptide, "ICAM-4," as well as polypeptide variants (including fragments and deletion, substitution, and addition analogs) thereof which display one or more ligand/receptor binding biological activities and/or immunological properties specific to ICAM-4. ICAM-4-specific ligand/receptor binding biological activities encompass interactions of both the ICAM-4 extracellular and cytoplasmic domains with other molecules (e.g., in processes of cell-cell adhesion and/or signal transduction). Preferred DNA sequences of the invention include genomic and cDNA sequences as well as wholly or partially chemically synthesized DNA sequences. A presently preferred polynucleotide is set out in SEQ ID NO: 1 and encodes rat species ICAM-4. Biological replicas (i.e., copies of isolated DNA sequences made *in vivo* or *in vitro*) of DNA sequences of the invention are contemplated. Also provided are autonomously replicating recombinant constructions such as plasmid and viral DNA vectors incorporating ICAM-4 sequences and especially vectors wherein DNA encoding ICAM-4 or an ICAM-4 variant is operatively linked to an endoge-nous or exogenous expression control DNA sequence.

According to another aspect of the invention, host cells, especially unicellular host cells such as procaryotic and eucaryotic cells, are stably transformed with DNA sequences of the invention in a manner allowing the desired polypeptides to be expressed therein. Host cells expressing such

ICAM-4 and ICAM-4 variant products can serve a variety of useful purposes. To the extent that the expressed products are "displayed" on host cell surfaces, the cells may constitute a valuable immunogen for the development of antibody substances specifically immunoreactive with ICAM-4 and ICAM-4

5   variants. Host cells of the invention are conspicuously useful in methods for the large scale production of ICAM-4 and ICAM-4 variants wherein the cells are grown in a suitable culture medium and the desired polypeptide products are isolated from the cells or from the medium in which the cells are grown.

Novel ICAM-4 of the invention may be obtained as isolates

10   from natural cell sources, but, along with ICAM-4 variant products, are preferably produced by recombinant procedures involving host cells of the invention. A presently preferred amino acid sequence for an ICAM-4 polypeptide is set out in SEQ ID NO: 2. The products may be obtained in fully or partially glycosylated, partially or wholly de-glycosylated, or non-

15   glycosylated forms, depending on the host cell selected for recombinant production and/or post-isolation processing. ICAM-4 variants of the invention may comprise water soluble or insoluble monomeric, multimeric or cyclic ICAM-4 fragments which include all or part of one or more of the domain regions specified above and having a biological or immunological property of

20   ICAM-4 including, e.g., the ability to bind to a binding partner of ICAM-4 and/or inhibit binding of ICAM-4 to a natural binding partner. ICAM-4 variants of the invention may also comprise polypeptide analogs wherein one or more of the specified amino acids is deleted or replaced: (1) without loss, and preferably with enhancement, of one or more biological activities or

25   immunological characteristics specific for ICAM-4; or (2) with specific disablement of a particular ligand/receptor binding function. Analog polypeptides including additional amino acid (e.g., lysine or cysteine) residues that facilitate multimer formation are contemplated.

Also comprehended by the present invention are antibody

30   substances (e.g., monoclonal and polyclonal antibodies, antibody fragments,

single chain antibodies, chimeric antibodies, CDR-grafted antibodies and the like) and other binding proteins (*e.g.*, polypeptides and peptides) which are specific (*i.e.*, non-reactive with the ICAM-1, ICAM-2, and ICAM-R intercellular adhesion molecules to which ICAM-4 is structurally related) for

5   ICAM-4 or ICAM-4 variants. The invention also comprehends hybridoma cell lines which specifically secrete monoclonal antibodies of the invention. Presently preferred hybridomas of the invention include those designated 127A, 127H, 173E, 179I, and 179H. Antibody substances can be developed using isolated natural or recombinant ICAM-4 or ICAM-4 variants or cells

10   expressing such products on their surfaces. Binding proteins of the invention are additionally useful for characterization of binding site structure(s) (*e.g.*, epitopes and/or sensitivity of binding properties to modifications in ICAM-4 amino acid sequence).

         Binding proteins are useful, in turn, in compositions for

15   immunization as well as for purifying polypeptides of the invention and identifying cells displaying the polypeptides on their surfaces. They are also manifestly useful in modulating (i.e., blocking, inhibiting or stimulating) ligand/receptor binding biological activities involving ICAM-4, especially those ICAM-4 effector functions involved in specific and non-specific immune

20   system responses. Anti-idiotypic antibodies specific for anti-ICAM-4 antibody substances and uses of such anti-idiotypic antibody substances in modulating immune responses are also contemplated. The invention further provides methods of screening for neuropathology in an individual comprising the steps of: a) obtaining a fluid sample from the individual; b) contacting the

25   sample with an antibody specifically immunoreactive with ICAM-4; c) quantitating the level of ICAM-4/antibody binding in the sample; and d) comparing the level of ICAM-4/antibody binding in the sample to the level of ICAM-4/antibody binding in individuals (controls) known to be free of the neuropathology. Assays for the detection and quantification of ICAM-4 on

30   cell surfaces and in body fluids, such as serum or cerebrospinal fluid, may

involve, for example, a single antibody substance or multiple antibody substances in a "sandwich" assay format. In detecting ICAM-4 in a body fluid, antibodies of the invention are also useful for assessing the occurrence of neuropathologies which can be correlated to increased levels of circulating

5   ICAM-4. Such neuropathologies include, but are not limited to, cerebral ischemia (i.e., stroke) resulting from various disorders including, for example, thrombosis, embolism, cerebral aneurysmal hemorrhage, vasospasm, and the like. Quantitation of circulating ICAM-4 can also distinguish between various forms of epilepsy and may also permit determination of the stage of AIDS

10   progression. Still other neurodegenerative disorders for which measurement of circulating ICAM-4 can be useful for diagnosis include various forms of Alzheimer's disease and other cortical dementias (such as Pick's disease, diffuse cortical Lewy body disease, and frontal lobe degeneracy), subcortical dementias (including Parkinson's disease, Huntington's disease, and

15   progressive supranuclear), a number of the primary psychiatric disorders (such as depression, schizophrenia and psychosis), as well as nongenetic dementias arising from, for example, infections, vasculitis, metabolic and nutritional disorders (e.g., thyroid, vitamin B12 deficiency), vascular disorders (multiple infarct, lacunar state, Binswanger's disease), toxic encephalopathies (e.g.,

20   exposure to carbon monoxide, heavy metals or other industrial pollutants) and tumors.

     The scientific value of the information contributed through the disclosures of DNA and amino acid sequences of the present invention is manifest. As one series of examples, knowledge of the sequence of a cDNA

25   for ICAM-4 makes possible the isolation by DNA/DNA hybridization of genomic DNA sequences encoding ICAM-4 and specifying ICAM-4 expression control regulatory sequences such as promoters, operators and the like. DNA/DNA hybridization procedures carried out with DNA sequences of the invention and under stringent conditions are likewise expected to allow

30   the isolation of DNAs encoding allelic variants of ICAM-4, other structurally

related proteins sharing one or more of the biological and/or immunological properties specific to ICAM-4, and proteins homologous to ICAM-4 from other species. DNAs of the invention are useful in DNA/RNA hybridization assays to detect the capacity of cells to synthesize ICAM-4. Also made

5 available by the invention are anti-sense polynucleotides relevant to regulating expression of ICAM-4 by those cells which ordinarily express the same. As another series of examples, knowledge of the DNA and amino acid sequences of ICAM-4 makes possible the generation by recombinant means of ICAM-4 variants such as hybrid fusion proteins (sometimes referred to as "immuno-

10 adhesions") characterized by the presence of ICAM-4 protein sequences and immunoglobulin heavy chain constant regions and/or hinge regions. See, Capon et al., Nature, 337: 525-531 (1989); Ashkenazi et al., P.N.A.S. (USA), 88: 10535-10539 (1991); and PCT WO 89/02922, published April 6, 1989. ICAM-4 variant fusion proteins may also include, for example, selected

15 extracellular domains of ICAM-4 and portions of other cell adhesion molecules.

DNA of the invention also permits identification of untranslated DNA sequences which specifically promote expression of polynucleotides operatively linked to the promoter regions. Identification and use of such

20 promoter sequences are particularly desirable in instances, for example gene transfer, which can specifically require heterologous gene expression in a limited neuronal environment. The invention also comprehends vectors comprising promoters of the invention, as well as chimeric gene constructs wherein the promoter of the invention is operatively linked to a heterologous

25 polynucleotide sequence and a transcription termination signal.

The DNA and amino acid sequence information provided by the present invention also makes possible the systematic analysis of the structure and function of ICAM-4 and definition of those molecules with which it will interact on extracellular and intracellular levels. The idiotypes of anti-ICAM-

30 4 monoclonal antibodies of the invention are representative of such molecules

and may mimic natural binding proteins (peptides and polypeptides) through which ICAM-4 intercellular and intracellular activities are modulated or by which ICAM-4 modulates intercellular and intracellular events. Alternately, they may represent new classes of modulators of ICAM-4 activities. Anti-idiotypic antibodies, in turn, may represent new classes of biologically active ICAM-4 equivalents. *In vitro* assays for identifying antibodies or other compounds that modulate the activity of ICAM-4 may involve, for example, immobilizing ICAM-4 or a natural ligand to which ICAM-4 binds, detectably labelling the nonimmobilized binding partner, incubating the binding partners together and determining the effect of a test compound on the amount of label bound wherein a reduction in the label bound in the presence of the test compound compared to the amount of label bound in the absence of the test compound indicates that the test agent is an inhibitor of ICAM-4 binding.

The DNA sequence information provided by the present invention also makes possible the development, by homologous recombination or "knockout" strategies [see, *e.g.*, Kapecchi, *Science, 244*: 1288-1292 (1989)], of rodents that fail to express a functional ICAM-4 protein or that express a variant ICAM-4 protein. Such rodents are useful as models for studying the activities of ICAM-4 and ICAM-4 modulators *in vivo*.

DETAILED DESCRIPTION OF THE INVENTION

The disclosures of parent U.S. Patent Application Serial No. 08/102,852, filed August 5, 1993, are specifically incorporated by reference. The examples of that application address, *inter alia*: design and construction of oligonucleotide probes for PCR amplification of ICAM related DNAs; use of the probes to amplify a human genomic fragment homologous to, but distinct from DNAs encoding ICAM-1 and ICAM-2; screening of cDNA libraries with the genomic fragment to isolate additional ICAM-R coding sequences; screening of cDNA libraries to isolate a full length human cDNA sequence encoding ICAM-R; characterization of DNA and amino acid

sequence information for ICAM-R, especially as related to ICAM-1 and ICAM-2; development of mammalian host cells expressing ICAM-R; assessment of indications of ICAM-R participation in adhesion events involving CD18-dependent and CD18-independent pathways; inhibition of cell adhesion to ICAM-R by ICAM-R-derived peptides; expression of variants of ICAM-R; preparation and characterization of anti-ICAM-R antibodies and fragments thereof; mapping of ICAM-R epitopes recognized by anti-ICAM-R monoclonal antibodies; assessment of the distribution and biochemical characterization of ICAM-R and RNA encoding the same; assessment of ICAM-R in homotypic cell-cell adhesion and immune cell activation/-proliferation; characterization of ICAM-R monoclonal antibodies; and assessment of differential phosphorylation and cytoskeletal associations of the cytoplasmic domain of ICAM-R. Also disclosed was the identification of a rodent ICAM-encoding DNA that, at the time, appeared to be the rat homolog of human ICAM-R, and the use of this DNA to construct and express DNAs encoding glutathione-S-transferase fusion proteins. The detailed description of how this rodent DNA was identified can be found in the parent application (U.S.S.N. 08/102,852) in Example 6, and is reproduced herein as Example 1. As more of the rodent ICAM-coding sequence was identified, it became apparent that the rodent ICAM DNA did not encode a rat species homolog of human ICAM-R, but, in fact, encoded a novel ICAM polypeptide, herein named ICAM-4. In order to appreciate the events which led to the identification of ICAM-4, a chronology is provided which is followed by a detailed description of the invention.

A first rodent genomic ICAM-4 sequence was identified which encoded a region homologous to domain 2 (herein SEQ ID NO: 3, and SEQ ID NO: 23 of U.S.S.N. 08/102,852) of human ICAM-R (herein as SEQ ID NO: 4). A second, overlapping genomic DNA (herein SEQ ID NO: 5, and SEQ ID NO: 26 of U.S.S.N. 08/102,852) was also identified which encoded both the domain 2 region of SEQ ID NO: 3, and sequences for ICAM-1.

Using SEQ ID NO: 3 as a probe, a rodent spleen cDNA (herein SEQ ID NO: 6, and SEQ ID NO: 25 in U.S.S.N. 08/102,852) was identified which encoded domains 2 through 5 as well as a fifth domain not previously observed as an ICAM domain. At this time, these newly identified rodent DNAs appeared to encode a rodent homolog of human ICAM-R, however alignment of 3´ regions of these DNAs with other ICAMs proved difficult.

The subsequent isolation of a 1 kb cDNA clone from a rat spleen library, and amplification of an RT-PCR fragment indicated that a portion of both the cDNA and genomic clones had not been sequenced. Another RT-PCR amplification product (SEQ ID NO: 7) confirmed this omission. It was determined that a fragment of 177 bp was excised from the genomic and cDNA clones by *Eco*RI digestion of the clones to isolate these sequences from λ phage for DNA sequencing studies. Reanalysis of SEQ ID NOs: 5 and 6 in light of these other sequences permitted identification of more accurate and complete sequences for the originally isolated genomic and cDNA clones, presented in corrected form herein as SEQ ID NOs: 8 and 9.

In order to identify a complete coding sequence for ICAM-4, a rat brain cDNA (SEQ ID NO: 10) was isolated, and 5´ end sequence determined by 5´ rapid amplification of cDNA ends (5´ RACE), the amplification product set forth in SEQ ID NO: 11. Combining information from the RT-PCR clone (SEQ ID NO: 7), the brain cDNA (SEQ ID NO: 10) and the RACE amplification product (SEQ ID NO: 11) permitted identification of the complete coding sequence for ICAM-4 (SEQ ID NO: 1).

The present invention is thus illustrated by the following examples. More particularly, Example 1 addresses cloning of a partial rodent ICAM-4 DNA. Example 2 describes Northern blot analysis of rodent ICAM-4 transcription. Example 3 describes isolation of a full length rodent ICAM-4 cDNA. Example 4 relates the *in situ* hybridization of rodent ICAM-4 in brain tissue. Example 5 addresses generation of ICAM-4 fusion proteins in prokaryotes. Example 6 describes production of monoclonal antibodies

- 14 -

specific for rat ICAM-4/GST fusion proteins. Example 7 describes expression
of soluble rat ICAM-4 proteins in a baculovirus expression system. Example
8 addresses production of monoclonal antibodies specific for rat ICAM-4
expressed in a baculovirus system. Example 9 describes immunocytochemical
analysis of rat ICAM-4 expression. Example 10 relates cloning of a human
genomic ICAM-4-encoding DNA. Example 11 addresses cloning of a human
ICAM-4-encoding cDNA. Example 12 describes Northern analysis of human
ICAM-4 expression. Example 13 describes generation of human ICAM-
4/GST fusion proteins. Example 14 addresses production of monoclonal
antibodies immunospecific for human ICAM-4. Example 15 describes
development of a capture assay for determining the concentration of soluble
ICAM-4 in a particular fluid. Example 16 applies the capture assay method
in assessing ICAM-4 concentration in the serum of stroke patients. Example
17 relates to assessment of ICAM-4 transcription in a rat epilepsy model.
Example 18 describes measurement of circulating ICAM-4 concentration as
an assessment of various neurodegenerative disorders. Example 19 addresses
cloning of a promoter region for human ICAM-4.

EXAMPLE 1
Cloning of Rat ICAM-Related DNA
A. _Isolation of a Rat Genomic ICAM-Related Domain 2 DNA_

A rat genomic library constructed in λ EMBL3 was screened
a with [^{32}P]-labeled probe generated by PCR from DNA encoding human
ICAM-3 domain 2 The sequence of the probe is set forth in SEQ ID NO: 12.
Library plaques were transferred to Hybond N+ nylon membranes
(Amersham, Arlington Heights, IL). Screening of all cDNA and genomic
libraries was performed according to standard protocols. Prehybridization and
hybridizations were carried out in a solution of 40-50% formamide, 5X
Denhardt's, 5X SSPE and 1.0% SDS at 42°C. Probes ([^{32}P]-labeled) were
added at a concentration of 10^5-10^6 cpm/ml of hybridization solution.

Following 16-18 hours of hybridization, nylon membranes were washed extensively at room temperature in 2X SSPE with 0.1% SDS and subsequently exposed to X-ray film at -80°C overnight. Positive plaques were subjected to one or more rounds of hybridization to obtain clonal phage. DNA prepared

5   from lysate of the positive clones was subcloned into pBS+ and sequenced.

A first genomic clone encoding a rat ICAM-related domain 2 was identified that was determined to be homologous to domain 2 regions in other ICAM family members (see for example, Table 1 of U.S. Patent Application Serial No. 08/102,852), yet was distinct from the previously

10   reported nucleotide sequences for rat ICAM-1 [Kita, *et al.*, *Biochem.Biophys.-Acta 1131*:108-110 (1992)] or mouse ICAM-2 [Xu, *et al.*, *J.Immunol. 149*:2560-2565 (1992)]. The nucleic acid and deduced amino acid sequences for this clone were disclosed in the co-pending parents to the present application as purportedly variant forms of rat ICAM-R and were set forth as

15   SEQ ID NOs: 23 and 24, respectively, in U.S.S.N. 08/102,852. Herein, these same sequences are set out in SEQ ID NOs: 3 and 13, respectively.

A second, overlapping clone was also identified with the same probes and was determined to contain the ICAM domain 2 sequence of SEQ ID NO: 3 and 5´ DNA encoding at least part of rat ICAM-1. The nucleic

20   acid sequence for this clone was set forth in the co-pending parent to the present application as SEQ ID NO: 26 and is set forth herein as SEQ ID NO: 5. This second clone indicated that the ICAM-related gene fragment of the first clone and the gene encoding rat ICAM-1 are located on the same rat chromosome within 5 kb of each other.

25   B.   Isolation of Rat ICAM-Related cDNA

In order to identify a more complete protein coding sequence for the ICAM-related polypeptide, [^{32}P]-labeled DNA encoding the domain 2 sequence from the rat genomic clone identified in Section A (SEQ ID NO: 3), *supra*, was used to screen a number of cDNA libraries from various rat

and mouse cell types, including rat macrophage (Clontech, Palo Alto, CA), peripheral blood lymphocyte (PBL) (Clontech), T cell (constructed in-house), and spleen (Clontech), and mouse PBL (Clontech), T cell (constructed in-house), and B cell (constructed in-house).

A single clone was identified in a rat spleen cDNA library (Clontech) which contained five Ig-like domains, four of which were homologous to domains 2 through 5 in both ICAM-1 and ICAM-R. Moreover, this clone included 3´ DNA encoding an apparent fifth Ig-like domain which had not been previously identified in any other ICAM polypeptide. In addition, the clone contained an unusual 3´ sequence subsequently determined to be a partial intron (discussed *infra*) located between domains 4 and 5, suggesting that the clone was the product of an immature or aberrantly spliced transcript. The presence of the unique domain and the determination that the 3´ region did not properly align with other known ICAMs suggested that the ICAM-related DNA potentially encoded a novel rat ICAM polypeptide. The nucleic acid sequence for this clone was set forth in the parent to the present application as SEQ ID NO: 25; herein the nucleic acid sequence for this spleen cDNA clone is set forth in SEQ ID NO: 6.

C.   Re-analysis of Rat cDNA and Genomic DNAs

Subsequent to the August 5, 1993 filing of U.S. Patent Application Serial No. 08/102,852, it was determined that the partial rat spleen cDNA clone (SEQ ID NO: 25 in the parent and SEQ ID NO: 6 herein) and the rat liver genomic clone (SEQ ID NO: 26 of the parent and SEQ ID NO: 5 herein) were missing an internal 177 bp EcoRI fragment that was part of each of these clones but lost in a subcloning step when the library inserts were removed from the λ vector with *Eco*RI digestion and ligated into a sequencing vector. The observation that the cDNA and genomic clones might be missing a coding fragment became apparent upon alignment of the rat

genomic and cDNA sequences with various RT-PCR amplification products, including SEQ ID NO: 7, which revealed a gap in the rat sequence.

   Subsequent isolation and sequence alignment of a cDNA from a spleen library using the spleen cDNA clone (SEQ ID NO: 6) as a probe provided a first indication that a portion of the spleen cDNA and genomic clones were not sequenced. Further confirmation of this idea became apparent upon amplification of an RT-PCR fragment, spanning domains 3 through 5, using a 5´ primer (RRD3 5´Xho, containing a 5´ XhoI restriction site to facilitate cloning) set out in SEQ ID NO: 14, and a 3´ primer (RRD5 3´Hind, containing a HindIII site to facilitate cloning) set out in SEQ ID NO: 15.

   GAACTCGAGGCCATGCCTCCACTTTCC (SEQ ID NO: 14)

   CCATAAGCTTTATTCCACCGTGACAGCCAC (SEQ ID NO: 15)

Alignment of these two DNAs clearly revealed that the cDNA and genomic clones had lost a fragment prior to sequencing; this idea was further supported following sequencing of the RT-PCR DNA discussed *infra*. It was concluded that restriction digestion with *Eco*RI to remove the cDNA and genomic fragments prior to sequencing resulted in the excision of a 177 bp fragment that was not detected visually in the agarose gel separation of the clones from the λ phage sequences. Subsequent sequence analysis confirmed the location of two *Eco*RI sites flanking a 177 bp fragment in both of the original clones.

   The 177 bp *Eco*RI fragment is situated between nucleotides 719 and 896 in the rat partial cDNA clone as set out in SEQ ID NO: 9 and between nucleotides 2812 and 2989 in the partial genomic clone as set out in SEQ ID NO: 8.

D.   DNA Isolated by RT-PCR Clone

   RT-PCR was utilized to generate more complete sequence information for the rat ICAM-related gene. Sequence information from the genomic clone (SEQ ID NO: 3) was used to design sense primers complementary to a region 5´ of the protein coding region, as determined from the

cDNA clone, and antisense primers designed complementary to coding sequences and regions 3´ to the coding sequence in the cDNA clone (SEQ ID NO: 6).

Template cDNA for PCR reactions was prepared as follows. Approximately 2 μg of poly A$^+$RNA isolated from rat spleen cells was denatured by heating at 65°C in a 10 μl volume. Following denaturation, 0.1 μl RNasin (Invitrogen, San Diego, CA), 5 μl 5X RTase Buffer (BRL, Bethesda, MD), 2 μl random hexamer (pd(N)6 at 100 μg/ml) (Pharmacia, Piscataway, NJ), 6 μl dNTPs (2 mM each) and 2 μl AMV RTase (BRL) were added and the reaction was incubated at 42°C for 60-90 min. Reactions were stored at -20°C until needed.

An initial series of experiments was conducted to identify oligonucleotides primer pairs that produced an amplification product in PCR reactions using rat spleen cDNA as the template. Various 5´ sense primers were paired in PCR with a 3´ primer which was designed to be complementary to an internal, coding sequence; the 3´ primer was designated RRD2 3-1 and is set forth in SEQ ID NO: 16.

AACGTGCGGAGCTGTCTG   (SEQ ID NO: 16)

(In the ultimately isolated RT-PCR product, SEQ ID NO: 7, *infra*, primer RRD2 3-1 corresponded to nucleotides 719 through 736.) Similarly, various 3´ antisense primers were paired with a 5´ primer designed complementary to another internal, coding sequence; the 5´ primer in these reactions was designated RGen3900S and is set forth in SEQ ID NO: 17.

ACGGAATTCGAAGCCATCAACGCCAGG   (SEQ ID NO: 17)

(In SEQ ID NO: 7, *infra*, primer RGen3900S corresponded to nucleotides 1719 through 1736.) Based on the size of the amplification products and the ability of these products to hybridize with the partial cDNA clone, one pair of primers was determined to be most efficient and was used in subsequent PCR amplifications. The 5´ primer was designated RGen780S (SEQ ID NO: 18) and the 3´ primer was designated RGen4550AS (SEQ ID NO: 19).

CATGAATTCCGAATCTTGAGTGGGATG   (SEQ ID NO: 18)

ATAGAATTCCTCGGGACACCTGTAGCC   (SEQ ID NO: 19)

(In SEQ ID NO: 7, *infra*, primer RGen780S corresponded to nucleotides 1 through 18, and primer RGen4550AS corresponded to nucleotides 2197

5 through 2214.)

This primer pair was used in PCR under a variety of conditions to optimize amplification. A total of 15 different PCR buffers that varied in pH and Mg^{++} concentration were used at two different annealing tempera-tures, and a sample of the product from each reaction was separated on a 1%

10 agarose gel. Because no amplification product could be detected by visual inspection of the ethidium bromide stained gel from any of the reaction conditions, more sensitive Southern hybridization was employed to detect the PCR products.

Aliquots of the amplified DNA were separated by electrophore-

15 sis, transferred to a Hybond N+ nylon membrane using conventional Southern blotting wicking techniques, and hybridized with the entire rat cDNA which was $[^{32}P]$-labeled. Hybridization conditions were essentially as described for the library screening procedure in Section A, *supra*. Autoradiography indicated that a small amount of DNA of approximately 2.2 kb had been

20 generated in two of the reactions, and the remainder of the amplification product from the two reactions was separated on an agarose gel. The 2.2 kb region was eluted from the gel, even though no band was evident upon visual inspection, and used as a template in another PCR reaction using the same primers (SEQ ID NOs: 18 and 19), Tris-HCl buffer, pH 8.0, containing 1

25 mM Mg^{++}, and 55°C annealing temperature. The amplification product from the secondary PCR was visible in the gel and was eluted and cloned into a pBS+ plasmid (Stratagene, La Jolla, CA) for sequence analysis.

The resulting RT-PCR clone was determined to contain 2214 bp as set forth in SEQ ID NO: 7. The clone encoded domains 2 through 6

30 found in the rat spleen cDNA clone, an additional amino terminal domain 1,

an additional carboxy terminal domain 7, and 164 bp of what appeared to be a further carboxy terminal domain 8. Immediately 5′ to domain 1 was an additional 144 bp sequence presumed to have been derived from an intron between the leader and the first domain. This clone did not contain a 5′ leader sequence or 3′ transmembrane and cytoplasmic regions. In addition to the previously identified domain 6 in the spleen cDNA clone, the 7th and 8th domains in the RT-PCR clone supported the hypothesis that this clone was a novel rodent ICAM.

EXAMPLE 2

Northern Blot Analysis

In order to further investigate the possibility that the ICAM-related clones identified in Example 1 encoded a novel ICAM polypeptide as suggested by the unique Ig-like domains, tissue specific expression was examined by Northern blot analysis to permit comparison with the previously reported expression patterns of human ICAMs [ICAM-1, Dustin, *et al.*, *J.Immunol.* 137:245-254 (1986); ICAM-2, Staunton, *et al.*, *Nature* 339:61-64 (1989); ICAM-R, de Fourgerolles and Springer, *J.Exp.Med.* 175:185-190 (1992)].

Total cellular RNA from rat lung, brain, spinal cord, liver, digestive tract, thymus, lymph nodes, and spleen was prepared using STAT60 RNA isolation reagents (Tel-test "B", Inc, Friendswood, Texas) according to the manufacturer's suggested protocol. Poly A^+ RNA was purified from total RNA using oligo dT cellulose columns. Approximately 5 μg of RNA derived from each tissue was separated on a 1% formaldehyde agarose gel, and transferred to hybond-C nitrocellulose membranes (Amersham).

A fragment of the rat spleen cDNA from Example 1 corresponding to domains 2 through 4 (nucleotides 1 through 724 in SEQ ID NO: 6) was subcloned into pBluescript SK$^+$ (Stratagene) and an antisense riboprobe was generated by *in vitro* transcription using ^{32}P-labeled UTP and approximately 500 ng of linearized template according to a manufacturer's

(Boehringer Mannheim, Indianapolis, IN) suggested protocol. The membrane-bound RNA was prehybridized in a solution containing 50% formamide, 5X SSC, 1X PE (50 mM Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 0.2% polyvinylpyrrolidone, 0.2% ficoll, 5 mM EDTA, 1% SDS) and 150 μg/ml denatured salmon sperm DNA. The radiolabeled probe was denatured by boiling and added to the prehybridization solution to a final concentration of 1×10^6 cpm/ml. Hybridization was allowed to proceed for 16-18 hours at 65°C. The membranes were then washed at 65°C in 2X SSC containing 0.1% SDS and subsequently exposed to X-ray film for 3-16 hours.

The Northern blot analysis indicated that the ICAM-related cDNA identified in Example 1 was expressed only in rat brain, a tissue specificity not previously reported for any other ICAM polypeptides. This expression pattern, in combination with the unique Ig-like domains not known to exist in other ICAM polypeptides, indicated that the ICAM-related clone was a novel member of the ICAM family of proteins, and was named ICAM-4.

The fact that the initially identified cDNA clones were detected in a rat spleen library suggested that a subset of cells in the spleen may express ICAM-4 at low levels. However, a properly spliced clone could not be detected in numerous hemopoietic cDNA libraries which led to doubt if ICAM-4 protein is actually expressed in tissue other than brain. One explanation for the detection of ICAM-4 cDNA in spleen is that the sensitivity of PCR may have amplified a trace amount of transcript even though these tissues do not express the encoded protein.

EXAMPLE 3
Isolation of Full Length Rat ICAM-4 cDNA

A.   Identification of a Rat Brain cDNA Clone

In view of the tissue specific expression of ICAM-4, brain tissue mRNA was utilized in an attempt to isolate a full length cDNA

encoding ICAM-4. Two probes, one complementary to domains 1 through 2 and a second complementary to domains 3 through 5 of the spleen cDNA clone identified in Example 1 (SEQ ID NO: 7), were radiolabeled and used to screen a rat brain cDNA library in λgt10 which was previously constructed in-house. Hybridization conditions were as described in Example 1, and positive plaques were subjected to one or more rounds of screening to obtain clonal phage.

Nine positive clones were identified, two of which hybridized to both probes. The longest of the two clones, designated clone 7, contained 2550 bp encoding four of the five Ig-like domains found in the probe cDNA. In addition, clone 7 encoded four other Ig-like domains not found in the probe. Putative transmembrane and cytoplasmic domains were identified which were followed by a stop codon, a poly-adenylation signal, and a poly A tail. Clone 7 was lacking at least one 5´ Ig-like domain as determined by comparison to the RT-PCR clone (SEQ ID NO: 7), and also lacked a leader sequence; re-screening of the library did not yield any longer clones which contained these sequences. The nucleic acid sequence for clone 7 is set forth in SEQ ID NO: 10.

B.   Determination of the 5´ End

In order to isolate domain 1 and other 5´ sequences, a PCR technique termed 5´ Rapid Amplification of cDNA Ends (RACE) [PCR Protocols: A Guide to Methods and Applications, Innis, et al., (eds) Academic Press: New York (1990) pp:28-38] was employed using a 5´ RACE kit (Clontech). This technique utilizes an internal primer paired with a second primer complementary to an adapter sequence ligated to the 5´ end of cDNA library molecules. PCR with this primer pair will therefore amplify and facilitate identification of the intervening sequences. Overlapping sequence information can then be used to generate a complete sequence of the gene.

RACE-ready cDNA from rat brain (supplied with kit) was used in a PCR with the kit oligonucleotide and an antisense primer based on an internal ICAM-4 sequence. The 3´ antisense primer, designated Spot714AS, was designed according to an ICAM-4 domain 4 sequence and is set forth in SEQ ID NO: 20.

CARGGTGACAAGGGCTCG   (SEQ ID NO: 20)

The amplification product resulting from this primer pair was subsequently subjected to a secondary PCR using the same 5´ kit primer paired with a 3´ primer complementary to a region in ICAM-4 domain 1. The second 3´ primer was designated RRACE2 and is set forth in SEQ ID NO: 21.

TATGAATTCAGTTGAGCCACAGCGAGC   (SEQ ID NO: 21)

Each primer used in the secondary PCR contained an *Eco*R1 site to facilitate cloning of the resulting amplification products into pBS$^+$ (Stratagene). The resulting plasmid DNA which contained the 5´ end of the gene was identified by hybridization to a rat ICAM-4 domains 1 and 2 probe, corresponding to nucleotides 1 through 736 in SEQ ID NO: 7. Partial sequence information for domain 1 and the hydrophobic leader was determined from the resulting amplification product.

The product from the 5´ RACE method was a DNA fragment 222 bp long containing 60 bp upstream of the initiating methionine residue, an 82 bp leader sequence, and an 80 bp sequence from domain 1. The amplification product is set forth in SEQ ID NO: 11.

C.   Full Length Sequence of Rat ICAM-4

A composite clone of the full length ICAM-4 was constructed from the sequence information derived from the 5´ RACE method (SEQ ID NO: 11), the RT-PCR clone (SEQ ID NO: 7) and the brain cDNA clone 7 (SEQ ID NO: 10). The full length gene for rat ICAM-4 was determined to contain 2985 bp with a single open reading frame encoding a deduced 917 amino acid protein. A putative Kozak sequence is located upstream of the

methionine residue in the leader sequence. A 27 amino acid hydrophobic leader sequence is followed by nine Ig-like domains, a transmembrane region and a 58 amino acid cytoplasmic tail. The composite ICAM-4 cDNA is set for in SEQ ID NO: 1, and the deduced amino acid sequence is set forth in
5   SEQ ID NO: 2.

Like other ICAM polypeptides, ICAM-4 contains extracellular, transmembrane, and cytoplasmic domains. In the extracellular domain, the amino terminus of ICAM-4 is a leader sequence comprising amino acids 1 through 27 which is followed by nine immunoglobulin (Ig)-like domains, a
10   characteristic unique to ICAM-4 in that ICAM-1, ICAM-2, and ICAM-R contain five, two, and five extracellular Ig-like domain, respectively. In ICAM-4, domain 1 comprises amino acids 28 through 118; domain 2 comprises amino acids 119 through 224; domain 3 comprises amino acids 225 through 321; domain 4 comprises amino acids 322 through 405; domain 5
15   comprises amino acids 406 through 488; domain 6 comprises amino acids 489 through 569; domain 7 comprises amino acids 570 through 662; domain 8 comprises amino acids 663 through 742; and domain 9 comprises amino acids 743 through 830. Within each domain, a characteristic "loop" structure is formed by a disulfide bond between cysteine residues located generally at
20   opposite ends of the domain amino acid sequence. Other structural features of ICAM-4 include the transmembrane region comprising amino acids 831 through 859 and the cytoplasmic region comprising amino acids 860 through 917.

Comparison of amino acid sequence homology of each domain
25   in rat ICAM-4 with the other members of the ICAM family was limited to the corresponding sequences of human ICAM-1, ICAM-2, and ICAM-R since sequence information for all three rodent homologs has not been previously reported. In the first domain, the rodent ICAM-4 shows 21, 30, and 28 percent identity with human ICAM-1, ICAM-2, and ICAM-R, respectively.
30   The second domain is more conserved, with the amino acid percent identities

being 60, 42 and 62 with ICAM-1, -2, and -3, respectively. Domains 3-5 show percent identities of 48, 49, and 40 with ICAM-1 and 60, 59 and 29 respectively for ICAM-R. Interestingly, rat ICAM-4 domains 6 through 8 are most homologous with domain 5 (ranging from 29-42% identical), possibly arising from a gene segment duplication event. The ninth and final extracellular domain aligns poorly with other ICAM domains but has 22% identity with the 3rd and 6th domains of human VCAM-1, another member of the Ig family of protein which participate in cell adhesion. The cytoplasmic tail is 58 amino acids long. This is longer than the other members of the ICAM family wherein human ICAM-1, -2, and -3 contain 28, 26, and 37 amino acids, respectively. As with the ninth domain, rat ICAM-4 cytoplasmic tail is most homologous with the cytoplasmic tail of human VCAM-1, which contains only 19 amino acids. The membrane proximal 19 amino acids of rat ICAM-4 share 7 amino acid residues with VCAM-1 (37%).

Finally, functional binding to LFA-1 (CD11a/CD18) maps to the first domain in the ICAMs. Vonderheide *et al.*,[*J. Cell. Biol., 125*:215-222 (1994)] identified a sequence motif purportedly involved in integrin binding. Despite the relatively low homology between rat ICAM-4 and other ICAMs in domain 1, this binding sequence motif is conserved, suggesting that rat ICAM-4 may be a ligand for LFA-1 and perhaps other integrins.

EXAMPLE 4

In situ Hybridization in Brain Tissue

In order to localize the specific brain tissue which expressed ICAM-4, *in situ* hybridization with ICAM-4 domain 1 and ICAM-4 domains 3 through 4 anti-sense riboprobes was employed. The probes were labeled by *in vitro* transcription using ^{35}S-labeled UTP.

Frozen tissue sections of normal rat brain were fixed in 4% paraformaldehyde for 20 minutes, rinsed and dehydrated, and the fixed RNA denatured for 2 minutes in 2X SSC, 70% formamide at 70°C prior to

hybridization. Tissue sections were hybridized overnight at 50°C in a solution containing 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 10% dextran sulfate, 1X Denhardt, 0.5 mg/ml yeast RNA, 100 mM DTT and a probe concentration of 50,000 cpm/μl. Slides were washed once in 4X SSC, 10 mM DTT at room temperature for 60 minutes, once in 50% formamide, 2X SSC, 10 mM DTT at 60°C for 40 minutes, and once in each 2X SSC and 1X SSC for 30 minutes each at room temperature. Specificity of hybridization was determined in parallel experiments performed with the same protocol but also including a more stringent wash in 50% formamide, 1X SSC, 10 mM DTT at 60°C for 40 minutes. After washing, the slides were dipped in NTB2 emulsion (Kodak, Rochester, NY) and exposed from 2 to 21 days before being developed and counter-stained. Negative controls included sense probes generated from ICAM-4 domain 1 and ICAM-4 domain 3 through 4 sense riboprobes, in addition to a human immunodeficiency virus (HIV-1) riboprobe.

The signal detected in brain tissue was primarily localized in the gray matter with the strongest signal in the cerebral cortex and hippocampus. The hybridization profile was consistent with ICAM-4 expression primarily in cerebral neurons.

EXAMPLE 5

Generation of ICAM-4 fusion proteins

Rat ICAM-4/glutathione S-transferase (GST) fusion proteins were generated using the prokaryote expression vector pGEX (Pharmacia, Alameda, CA) in order to generate monoclonal antibodies against specific ICAM-4 polypeptide fragments.

PCR primers corresponding to the 5´ and 3´ ends of domain 1 and the 5´ and 3´ ends of domain 2 were used to amplify DNA fragments encoding the individual domains. The resulting fragments were separately cloned into an EcoRI site of pGEX-2T; DNA sequence analysis confirmed the

correct orientation and reading frame. Transformants were subsequently screened for their ability to produce fusion protein of the appropriate molecular weight.

Both ICAM-4 domain 1/GST and ICAM-4 domain 2/GST
5   fusion proteins remained in the insoluble fraction after the bacteria were lysed by sonication in PBS containing 1 % SDS. The insoluble protein fraction from 100 ml cultures were boiled in SDS loading dye and separated on a 10 % preparative polyacrylamide-SDS gel. The gel was stained in ice cold 0.4 M KCl and the fusion protein bands were excised. Fusion proteins were
10 electroeluted from the gel slices in dialysis tubing in buffer containing 25 mM Tris-HCl and 192 mM glycine. Approximate protein concentration was determined by OD_{280} and purity of the preparation was determined on SDS-PAGE stained with Coomassie blue.

EXAMPLE 6

15 **Production of Monoclonal Antibodies**
Against Rat ICAM-4/GST Fusion Proteins

Balb/c mice were immunized by subcutaneous injection with 40-50 μg ICAM-4 domain-2/GST fusion protein (described in Example 5) emulsified in Freund's complete adjuvant (FCA). Two weeks later, the mice
20 were again immunized by subcutaneous injection with the same protein, emulsified however in Freund's incomplete adjuvant. Two final intraperitone-al immunizations given two weeks after the second immunization included soluble antigen with no adjuvant given at two week intervals. Serum from each immunized mouse was assayed by ELISA for its ability to specifically
25 react with rat ICAM-4 produced by the baculovirus expression system described *infra*.

The spleen from mouse #1654 was sterilely removed and placed in 10 ml serum-free RPMI 1640. A single-cell suspension was formed by grinding the spleen tissue between frosted ends of two glass microscope slides
30 submerged in serum free RPMI 1640 (Gibco, Burlington, Ottawa, Canada)

supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cell suspension was filtered through a sterile 70-mesh Nitex cell strainer (Becton Dickinson, Parsippany, NJ), and washed twice with RPMI followed by centrifuging at 200 x g for 5 minutes. The resulting pellet from the final wash was resuspended in 20 ml serum-free RPMI. Thymocytes taken from three naive Balb/c mice were prepared in an identical manner.

      Prior to fusion, NS-1 myeloma cells were maintained in log phase growth in RPMI with 11% Fetalclone serum (FBS) (Hyclone Laboratories, Logan, Utah) for three days. Once harvested, the cells were centrifuged at 200 x g for 5 minutes, and the pellet was washed twice as described in the foregoing paragraph. After washing, the cell suspension was brought to a final volume of 10 ml in serum free RPMI. A 20 μl aliquot was removed and diluted 1:50 with serum free RPMI, and a 20 μl aliquot of this dilution was removed, mixed with 20 μl 0.4% trypan blue stain in 0.85% saline (Gibco), loaded onto a hemacytometer (Baxter Healthcare, Deerfield, IL) and the cells counted. Approximately 2.425 x 10^8 spleen cells were combined with 4.85 x 10^7 NS-1 cells, the mixture centrifuged and the supernatant removed. The resulting pellet was dislodged by tapping the tube and 2 ml of 50% PEG 1500 in 75 mM Hepes, pH 8.0, (Boehringer Mannheim, Indianapolis, IN) was added with stirring over the course of 1 minute. Subsequently, an additional 14 ml serum free RPMI was added over 7 minutes. The cell suspension was centrifuged at 200 x g for 10 minutes and the supernatant discarded. The pellet was resuspended in 200 ml RPMI containing 15% FBS, 100 μM sodium hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine (HAT) (Gibco), 25 units/ml IL-6 (Boehringer Mannheim) and 1.5 x 10^6 thymocytes/ml. The suspension was first placed in a 225 cm^2 flask (Corning, Essex, United Kingdom) at 37°C for four hours before being dispensed into ten 96-well flat bottom tissue culture plates (Corning) at 200 μl/well. Cells in the plates were fed on days 3, 4, 5, and 6 post fusion by aspirating approximately 100 μl

from each well with a 20 G needle (Becton Dickinson), and adding 100 μl/well plating medium described above except containing 10 units/ml IL-6 and lacking thymocytes.

5   The fusion plates were screened initially by antigen capture ELISA as follows. Immulon 4 plates (Dynatech, Cambridge, MA) were coated overnight at 4°C with 100 ng/well of either domain 1-GST or domain 2-GST fusion protein in 50 mM carbonate buffer. The plates were blocked with 100 μl/well 0.5% fish skin gelatin (Sigma, St. Louis, MO) in PBS for 30 minutes at 37°C. After blocking, the plates were washed 3X with PBS

10   containing 0.05% Tween 20 (PBST) and 50 μl/well of hybridoma supernatant from each fusion was added. After incubation at 37°C for 30 minutes, the plates were washed as described above, and 50 μl of a 1:3500 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Fc) (Jackson ImmunoResearch, West Grove, Pennsylvania) was added. Plates were again

15   incubated for 30 minutes and washed 4X with PBST. Substrate, 100 μl/well, consisting of 1 mg/ml o-phenylene diamine (Sigma) and 0.1 μl/ml 30% H_2O_2 in 100 mM citrate, pH 4.5, was added. The color reaction was allowed to proceed 10 minutes and quenched with the addition of 50 μl/well of 15% H_2SO_4. Absorbance at 490 nm was then determined on an automated plate

20   reader (Dynatech).

            Wells which were positive for domain 2-GST protein, but not for domain 1-GST protein, were then screened by ELISA against a Baculovirus supernatant (described *infra*). ELISA was performed as described above except that the Immulon 4 plates were initially coated overnight with

25   Baculovirus supernatant diluted 1:4 in 50 mM carbonate buffer. Three wells (103A, 103B and 103F) were cloned two to three times, successively, by doubling dilution in RPMI, 15% FBS, 100 μM sodium hypoxanthine, 16 μM thymidine, and 10 units/ml IL-6. Wells of clone plates were scored visually after 4 days and the number of colonies in the least dense wells was recorded.

30   Selected wells of each cloning were again assayed by ELISA after 7 to 10

days against either domain 1-GST protein and domain 2-GST protein, or Baculovirus supernatant.

The monoclonal antibodies produced by the hybridomas were isotyped by ELISA. Immulon 4 plates (Dynatech) were coated at 4°C with 50 μl/well goat anti-mouse IgA, IgG, or IgM (Organon Teknika, Durham, NC) diluted 1:5000 in 50 mM carbonate buffer, pH 9.6. Wells were blocked for 30 minutes at 37°C with 1% BSA in PBS, washed 3X with PBST. A 1:10 dilution of hybridoma culture supernatant (50 μl) was added to each plate, incubated, and washed as above. After removal of the last wash, 50 μl horseradish peroxidase-conjugated rabbit anti-mouse IgG_1, G_{2a}, G_{2b}, or G_3 (Zymed, San Francisco, CA) (diluted 1:1000 in PBST with 1% normal goat serum) was added. Plates were incubated as above, washed 4X with PBST and 100 μl substrate, was added. The color reaction was quenched after 5 minutes with addition of 50 μl 15% H_2SO_4, and absorbance at 490 nm determined on a plate reader (Dynatech).

Results indicated that antibodies 103A, 103B, and 103F were all IgG_1 isotype. These antibodies were subsequently used in immunocytochemical analyses, Western blotting, and for purification of protein expressed in baculovirus.

EXAMPLE 7

Baculovirus Expression of Rat ICAM-4

A baculovirus expression system (Invitrogen) was used to generate soluble protein corresponding to domains 1 through 6 of ICAM-4. Because the leader sequence for ICAM-4 was not known at the time, the expression construct was made containing the coding sequence for ICAM-4 fused 3´ to the ICAM-1 leader sequence in proper reading frame. Specific details regarding construction of the ICAM-1/ICAM-4 expression plasmid is as follows.

Rat ICAM-1 DNA encoding the five Ig-like domains was amplified by PCR using primers which incorporated several features to facilitate construction of the fusion plasmid. The 5´ oligonucleotide primer included *Hind*III and *Bgl*II sites, in addition to a consensus Kozak sequence

5 upstream of the first methionine in the leader sequence. The 3´ oligonucleotide primer included a coding sequence for six histidines followed by a stop codon and a *Hind*III cloning site. The PCR amplification product was cloned into a *Hind*III-digested pBS⁺ vector and sequence analysis confirmed the appropriate construction. An internal *Sma*I site in the ICAM-1 leader

10 sequence and another *Sma*I site in the vector's multiple cloning region (3´ to ICAM-1 Ig-like domain 5) were digested which removed most of the ICAM-1 coding sequence. After these manipulations, the linearized, blunt-ended vector contained a portion of the upstream multiple cloning region (those restriction sites 5´ of the original *Hind*III site in the multiple cloning region),

15 the Kozak sequence and most of the ICAM-1 leader sequence.

The coding sequence for rat ICAM-4 domains 1 through 6 was amplified by PCR utilizing primers designed to permit cloning of this sequence into the linearized vector described above. The 5´ oligonucleotide primer included an *Eco*RV site and the codons needed to complete the

20 ICAM-1 leader sequence. The 3´ oligonucleotide primer included codons for six histidine residues, a stop codon, and *Hind*III and *Eco*RV restriction sites. The amplification product from this PCR was digested with *Eco*RV to produce a blunt-ended sequence which was then ligated into the blunt-ended *Sma*I-digested pBS⁺ linearized vector. The entire sequence containing the ICAM-1

25 leader sequence 5´ to the ICAM-4 domains 1 through 6 was removed from the construct with *Bgl*II and *Hind*III digestion and the purified ICAM-1/ICAM-4 fusion sequence cloned directly into a *Bgl*II/*Hind*III- digested pBluesac III vector (Invitrogen).

Protein production by the recombinant virus was assayed for by

30 ELISA, initially using immune sera from mice immunized with rat ICAM-4

domain-2/GST fusion protein described in Example 5. In later work, monoclonal antibodies generated from those mice were used to purify ICAM-4 protein produced by the recombinant baculovirus in SF9 cells.

EXAMPLE 8

5

Production of Monoclonal Antibodies
Against Baculovirus-expressed Rat ICAM-4

Rat ICAM-4 domains 1-6 were expressed in the baculovirus expression system as described in Example 7. The recombinant protein was purified using monoclonal antibody 103A (as described in Example 6).

10 Briefly, 30 mg of purified monoclonal 103A (in 100 mM sodium borate, 500 mM sodium chloride) were coupled to three grams of Activated Cyanogen Bromide Sepharose 4B (Pharmacia, Piscataway, NJ). Baculovirus supernatant containing recombinant rat ICAM-4 (domains 1-6) was loaded on the Sepharose column overnight at 4°C. The column was

15 washed in calcium- magnesium-free phosphate buffered saline (CMF-PBS) and bound material was eluted in 50 mM citric acid, 500 mM NaCl pH 4.0. The sample was neutralized with 1/10 volume Tris pH 10 and stored at -20°C. The purified protein separated on SDS-PAGE appeared greater than 90% pure and migrated at approximately 80 kD.

20 Mice were immunized with the purified recombinant rat ICAM-4 domains 1-6 protein in a similar manner as described in Example 6. The spleen from mouse #1945 was used for fusion #127. The fusion protocol was as described in Example 6. The fusion wells were screened by ELISA on the recombinant ICAM-4 protein. The secondary screen included immunocyto-

25 chemistry on rat brain sections (as below described in Example 9). Four additional antibodies specific for rat ICAM-4 were cloned out of this fusion: 127A, 127E, 127F and 127H. The immunocytochemical staining pattern of each antibody on rat brain sections was the same as observed with monoclonal antibody 103A (see Example 9). The monoclonal antibodies were tested for

30 their ability to bind the D1/GST and D2/GST fusion proteins (described in

Example 5). Monoclonal antibody 127A recognized the D1/GST fusion protein and 127H recognized the D2/GST fusion protein. These two distinct binding specificities along with the others that did not bind either GST protein suggest that at least 3 different epitopes were being recognized by the panel of antibodies. Hybridomas 127A and 127H were deposited May 31, 1995 and June 1, 1995, respectively, with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, and assigned Accession Numbers HB11905 and HB11911, respectively.

EXAMPLE 9
Immunocytochemistry of Rat ICAM-4 Expression

Immunocytochemistry with monoclonal antibody 103A was performed to localize the protein production within the rat brain.

A brain was harvested from a normal adult female Lewis rat, sagittally sectioned, and washed in RNase-free 1X PBS on ice for 30 min. The brain sections were then placed in Tissue Tek II cryomolds (Miles Laboratories, Inc., Naperville, IL) with a small amount of O.C.T. compound (Miles, Inc., Elkhart, IN). The brains were centered in the cryomold, the cryomold filled with OCT compound, then placed in a container with 2-methylbutane (Aldrich Chemical Company, Inc., Milwaukee, WI) and the container placed in liquid nitrogen. Once the tissue and OCT compound in the cryomold were frozen, the blocks were stored at -80°C until sectioning.

The tissue was sectioned at 6 μm thickness, adhered to Vectabond (Vector Laboratories, Inc., Burlingame, CA) coated slides and allowed to air-dry at room temperature overnight until use. The sections were fixed in ethyl ether (Malinckrodt, Paris, KY) for 5 minutes at room temperature. Once the slides were removed from the ether, the reagent was allowed to evaporate. Each tissue section was blocked with 150 μl 50% Normal rat serum (Sigma) and 2% bovine serum albumin (BSA) (Sigma) in 1X PBS (made with sodium phosphates only) for 30 minutes at room temperature.

After blocking, the solution was gently blotted from the sections and the purified supernatant antibody 103A (1.65 mg/ml) was diluted 1:10 in the blocking solution and 150 μl applied to each tissue section. The slides were placed in a humidity chamber and incubated at 4°C overnight.

5   The next day the antibody solution was blotted gently from the section and the slides washed three times in 1X PBS for four minutes in each wash. The excess PBS was aspirated from the slide and 100 μl of the secondary, rat anti mouse-biotin conjugated antibody (Jackson Immuno-Research Laboratories), diluted 1:100 in a solution of 10% normal rat serum

10   and 2% BSA in 1X PBS, applied to the tissues. Incubation was allowed to proceed for one hour at room temperature. The sections were washed two times in 1X PBS for four minutes in each wash, then 100 μl of ABC reagent from an Elite Rat IgG Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA), prepared according to the product insert, was applied to

15   each section. Incubation was allowed to proceed for 30 minutes at room temperature. After incubation, the slides were washed two times in 1X PBS (four minutes each wash) and 150 μl of Vector VIP Peroxidase Substrate Solution (Vector Laboratories, Inc., Burlingame, CA) applied to each section for approximately ten minutes. After color development, the sections were

20   rinsed under running tap water for five minutes, counterstained with Mayer's hematoxylin (Sigma) for 20 seconds, and rinsed again in gently running tap water for five minutes. The slides were dehydrated across a graded series of ethanols, passed through xylene and mounted with Accumount 60 (Stephens Scientific, Riverdale, NJ).

25   Immunohistochemistry of rat brain sections strained with mAb 103A indicated that rat ICAM-4 is expressed in the neuronal cells of the hippocampus. Staining pattern suggested that the protein might be limited to the neuronal processes (dendrites). Brain sections stained in a similar manner with an irrelevant antibody or second step reagent alone do not show the

30   distinct expression pattern seen with MAb 103A.

EXAMPLE 10

Cloning of a Human ICAM-4 Genomic DNA

During the cloning of rat ICAM-4 from genomic DNA, it was discovered that ICAM-4 and ICAM-1 were located within 5 kb of each other and this information was utilized in an attempt to clone the human homologue of ICAM-4.

Genome Systems Inc. (St. Louis, MO) amplified fragments in a human P1 library by PCR using human ICAM-1 domain 3 primers, a sense primer designed complementary to human ICAM-1 domain 3 (H-1/D3 S) and an antisense primer designed complementary to human ICAM-1 domain 3 (H-1/D3 AS). These primers are set forth in SEQ ID NOs: 22 and 23, respectively.

CCGGGTCCTAGAGGTGGACACGCA   (SEQ ID NO: 22)

TGCAGTGTCTCCTGGCTCTGGTTC   (SEQ ID NO: 23)

Two clones, designated 1566 and 1567, were identified and subjected to further analysis. Both P1 clones contained approximately 75-95 kb genomic DNA inserts. The clones were digested with *Bam*H1, separated with agarose gel electrophoresis, and blotted onto nylon membranes. Southern blots hybridization were performed under either low stringency (30% formamide) or high stringency (60% formamide) at 42°C with human ICAM-1, ICAM-3 or rat ICAM-4 radiolabeled probes; other constituents of the hybridization solution were as described in Example 1. The low stringency hybridization series was washed at room temperature in 2X SSPE containing 0.1% SDS. The high stringency hybridization was washed at 65°C in 0.2X SSPE containing 0.1% SDS. The washed membranes were exposed to X-ray film for 3.5 hours.

The differential hybridization indicated that human ICAM-1 was contained on a 5.5 kb *Bam*H1 fragment while human ICAM-3 was located on

a 4.0 kb and a 1.5 kb *Bam*H1 fragment. The human ICAM-1 and ICAM-R
fragments were subcloned into pBS+ and their identity confirmed by limited
sequence analysis.

A 7.0 kb *Bam*H1 fragment that hybridized with rat ICAM-4
under high stringency conditions was subcloned and further fragmented with
*Rsa*I restriction digestion.   Three *Rsa*I fragments that hybridized with rat
ICAM-4 were identified and their sequences determined. Based on homology
to rat ICAM-4, these fragments appeared to contain domains 2, 3, 4, 5 and
part of domain 6.

EXAMPLE 11

Cloning of a Human ICAM-4 cDNA

The fragments of genomic DNA corresponding to domains 2-5
of human ICAM-4 (described in Example 10) were used as probes to screen
a λgt10 Human hippocampus cDNA library (Clontech, Palo Alto, CA). The
library screening protocol was essentially as described in Example 1.

The longest human ICAM-4 clone (#18) that was found in that
library was only 992 bp (SEQ ID: 24) and corresponded to roughly the middle
of the predicted 3 kb gene. The 992 bp DNA insert from clone 18 (SEQ ID:
24) was used as a probe to screen a λZAPII human hippocampus cDNA
library (Stratagene, La Jolla, CA). This library yielded a number of positive
clones. The longest clone, #34, was 2775 bp (SEQ ID: 25).   Based on
alignments to the full length rat ICAM-4, it was predicted that this clone was
missing the leader sequence and approximately 30 bp at the 5´ end of domain
1. The poly A$^+$ tail at the 3´ end was missing, but the translation stop codon
was present.

A fragment of DNA corresponding to the first 3 domains
(nucleotides 1 to 840 in clone #34) was used as a probe to screen a λgt10
cDNA library derived from human cerebral cortex (Clontech, Palo Alto, CA).
One clone, 16-1 (SEQ ID: 26), was identified as having 1557 bp, and

included 39 bp of 5´ untranslated DNA, a leader sequence and sequence information through the fifth domain. Overlapping clones #34 (SEQ ID: 25) and 16-1 (SEQ ID: 26) were used to generate a composite of the full length human ICAM-4 sequence (SEQ ID: 27).

5   The full length gene is 2927 bp long and encodes a 924 amino acid protein. The ICAM-4 nucleotide sequence is set out in SEQ ID NO: 27 and the amino acid sequence is set out in SEQ ID NO: 28. Sequence alignment with the full length rat ICAM-4 gene (SEQ ID: 11) revealed an overall DNA sequence identity of 82% and 85% identity at the amino acid

10 level. The apparent 9 Ig like extracellular domain structure of the protein is conserved between rat and human. The leader sequence extends from amino acid 1 to 28; domain 1 from amino acid 29 to 117; domain 2 from amino acid 118 to 224; domain 3 from amino acid 225 to 320; domain 4 from amino acid 321 to 405; domain 5 from amino acid 406 to 488; domain 6 from amino acid

15 489 to 570; domain 7 from amino acid 571 to 663; domain 8 from amino acid 664 to 743; domain 9 from amino acid 744 to 837; the transmembrane region from amino acid 838 to 857 and the cytoplasmic tail from amino acid 858 to 924.

        Human ICAM-4 (HuICAM-4), in addition to being genetically

20 linked to ICAM-1 and ICAM-R, also showed certain common structural features that group them together as a family of molecules. A domain by domain alignment of HuICAM-4 with the other members of the ICAM family shows varying degrees of homology. Domain 1 amino acid sequence of HuICAM-4 is 21, 30 and 26% identical to domain 1 of ICAMs 1, 2 and 3

25 respectively. Domain 2 of HuICAM-4 is 61, 39 and 62% identical to ICAMs 1, 2 and 3 respectively. Domain 3 of HuICAM-4 is 50 and 65% identical to ICAMs 1 and 3 respectively. Domain 4 of HuICAM-4 is 54 and 64% identical to ICAMs 1 and 3 respectively. Domains 5-8 of HuICAM-4 are most homologous to the fifth domains of ICAM-1 and 3, with percent

30 identities ranging from 33-47 for ICAM-1 domain 5 and 21-31 for ICAM-R

domain 5. The ninth domain of HuICAM-4 aligns poorly with the other members of the ICAM family but is homologous to domains 3 (24% identical) and 6 (23% identical) of HuICAM-1.

EXAMPLE 12

Northern Analysis of Human ICAM-4 Expression

5

Two human multiple tissue Northern (MTN) blots were purchased from Clontech (Palo Alto, CA). These contained at least 2 μg of poly A^+ RNA from 16 different human tissues (as shown in Table 1) run on a denaturing formaldehyde 1.2% agarose gel and transferred to nylon membrane. The blots were prehybridized for three hours at 42°C in 10 ml of a solution containing 5X SSPE, 10X Denhardts solution, 50% formamide, 2% SDS and 100 μg/ml denatured salmon sperm DNA. The blots were hybridized in the above solution with a radiolabeled human ICAM-4 probe (clone #18, SEQ ID: 24) for 16 hours at 42°C. The following day, the blots were washed in a solution of 0.1X SSC/0.1% SDS at room temperature followed by a wash at 50°C. The blots were exposed to x-ray film at -80°C for 24 hours. Results of the analysis are shown below in Table 1.

Only the lane containing RNA from the brain hybridized to the ICAM-4 probe, giving a single band at approximately 3 kb. Longer exposure (five days) confirmed that only the brain had a detectable level of message. In order to determine if all lanes contained comparable amounts of RNA of comparable quality, the same blot was hybridized with a control β-actin probe. Blots were stripped of the ICAM-4 probe by treatment with a boiling solution of 0.1% SDS for 15 minutes, and subsequently probed in a similar manner with a β actin probe provided by the manufacturer. Except for minor variation in amounts, all lanes were shown to have good quality RNA.

TABLE 1

Northern Tissue Analysis of Human ICAM-4 Expression

	Tissue	PROBE	
		ICAM-4	β-Actin
5	Heart	-	+++
	Brain	+	++
	Placenta	-	+++
	Lung	-	+++
	Liver	-	+++
10	Skeletal muscle	-	++++
	Kidney	-	+++
	Pancreas	-	++
	Spleen	-	+++
	Thymus	-	+++
15	Prostate	-	+++
	Testis	-	+++
	Ovary	-	+++
	Small intestine	-	+++
	Colon	-	+++
20	Peripheral blood leukocyte	-	+++

Two additional Northern blots were purchased from Clontech that contained poly A^+ RNA from 16 different sub-regions of human brain (as shown in Table 2). Blots were probed in a manner similar to that used for tissue analysis and results are shown in Table 2. RNA quality and quantity loaded was checked by probing the blots with a β actin probe.

All of the regions that showed ICAM-4 expression are part of the telencephalon, with the exception of the thalamus which is considered part of the diencephalon. The hippocampus and cerebral cortex appeared to have the highest level of expression. The transcript size in all cases was the same, 3 kb. The exquisite tissue distribution of the ICAM-4 expression suggests that the promoter region may contain elements that confer the observed developmental and spatial expression of the gene product. The utility of such information may provide insight into the understanding of control of neural gene expression in general.

TABLE 2

Northern Brain Cell Type Analysis of Human ICAM-4 Expression

| | | PROBE | |
	Brain Region	ICAM-4	β-Actin
5	Amygdala	+ +	+ + +
	Caudate nucleus	+ +	+ + +
	Corpus callosum	+	+ + +
	Hippocampus	+ +	+ + +
	Hypothalamus	-	+ + +
10	Substantia nigra	-	+ + +
	Subthalamic nucleus	+	+ + +
	Thalamus	+	+ + +
	Cerebellum	-	+ + +
	Cerebral cortex	+ + +	+ + +
15	Medulla	-	+ + +
	Spinal cord	-	+ + +
	Occipital pole	+ +	+ + +
	Frontal lobe	+ +	+ + +
	Temporal lobe	+ +	+ + +
20	Putamen	+ +	+ + +

EXAMPLE 13

Generation of Human ICAM-4/IgG Fusion Proteins

Human ICAM-4/IgG1 fusion proteins expression plasmids were constructed to produce proteins for generating monoclonal antibodies and for

25 use in adhesion assays to identify potential ICAM-4 ligands. Two constructs were made; the first included DNA encoding domains 1-3 of HuICAM-4 and the second, domains 4-8. Both were linked to the Fc region of human IgG1 in vector pDCS1 that uses the cytomegalovirus (CMV) promoter to drive expression and the signal sequence from IgG4 to facilitate secretion of the

30 molecules.

PCR primers (shown below as SEQ ID NOs: 29-32) were designed to generate the necessary DNA fragments for sub-cloning. The "sense" primer for the 5´ end of domain 1 (HI4-D1(s), SEQ ID NO: 29) was designed to fill in 30 base pairs of domain 1 missing in clone #34. Primers

HI4-D1(S) (SEQ ID NO: 29) and HI4-D3(AS) (SEQ ID NO: 30) were used to generate a DNA fragment encoding domains 1-3 of human ICAM-4, corresponding to a region in SEQ ID NO: 1 from nucleotide 130 to nucleotide 996. Primers HI4-D3(S) (SEQ ID NO: 31) and HI4-D8(AS) (SEQ ID NO: 32) were used to generate a DNA fragment encoding domains 4-8 of human ICAM-4, corresponding to a region in SEQ ID NO: 30 from nucleotide 997 to nucleotide 2268. Each 5´ primer encoded a *BamH*I restriction site (GGATCC, indicated in bold below) and each 3´ (antisense) primer contained a *Xho*I site (CTCGAG, indicated in bold below) to facilitate subcloning 5´ to the IgG1 gene. All oligonucleotides contain spacer nucleotides (underlined, below) at the 5´ end to permit restriction digestion.

HI4-D1(S)   (SEQ ID NO: 29)
GTACTTACA**GGATCC**GCGGTCTCGCAG-
          GAGCCCTTCTGGGCGGACCTACAGCCTGCGTGGCGTTC

HI4-D3(AS)   (SEQ ID NO: 30)
ATTTCT**CTCGAG**GATGGTCACGTTCTCCCGG

HI4-D4(S)   (SEQ ID NO: 31)
ATTTCT**GGATCC**TACAGCTTCCCGGCACCACTC

HI4-D8(AS)   (SEQ ID NO: 32)
ATTTCT**CTCGAG**TTCCACGCCCACAGTGACGG

PCR reactions were carried out in a 50 μl volume using buffers supplied by Perkin Elmer with the AmpliTaq enzyme. Primers were added at a final concentration of 10 μg/ml and all four dNTPs were included at 2 mM. The reactions were continued through 30 cycles of denaturation (94°C for four minutes), annealing (50°C for two minutes) and extension (72°C for one minute). PCR products were visualized on agarose gels and an aliquot of

each reaction was used to subclone the PCR products into vector pCRII (Invitrogen, SanDiego, CA). Sequence analysis was performed to detect possible errors resulting from the amplification process and to confirm proper orientation. Appropriate clones were digested with *Bam*HI and *Xho*I and fragments separated with agarose gel electrophoresis. Purified fragments were ligated into a pDCS1 vector previously digested with *Bam*HI and *Xho*I and the resulting plasmids were sequenced to confirm proper orientation and reading frame.

Human ICAM-4 domains 1-3 and 4-8/IgG1 fusion proteins were obtained following transient transfection of the expression plasmids into COS7 cells and isolation of the secreted protein from the culture media. Transfection was carried out as follows. Adherent COS7 cells at approximately 50-60% confluence were washed with CMF-PBS and subsequently contacted with 10-15 μg of plasmid DNA in 7.5 ml serum-free DMEM media (Gibco, Gaithersburg, MD) containing 6 μl of 0.25 M chloroquine (Sigma, St. Louis, MO). An additional 7.5 ml of serum-free media containing 150 μl of DEAE dextran (50 mg/ml) (Sigma, St. Louis, MO) were added and the plates incubated 2-3 hours before the media was removed and replaced with 10% DMSO (Mallinckrodt, McGaw Park, Illinois) in PBS. After a one minute incubation, the DMSO solution was removed and replaced with fresh media containing 5% FBS. Each transfection included multiple plates, and media from cells expressing the same protein were pooled for protein isolation.

Media were collected every three days over the course of 3-4 harvests. Proteins were purified using a 0.4 - 0.8 ml Procep A column (Bioprocessing Ltd, England) pre-equilibrated with 35 mM Tris, 150 mM NaCl, pH 7.5. Culture media was loaded onto the column two times at a flow rate of less than 60 column volumes per hour. The column was washed one time with each of 20 column volumes of Tris/NaCl buffer, 20 column volumes of 0.55 M diethanolamine, pH 8.5, and 20 column volumes of 50 mM citric acid, pH 5.0. The fusion proteins were eluted into one ml fractions

using 50 mM citric acid pH 3.0 and each fraction was neutralized with 1/10 volume 1 M Tris, pH 9.5. Protein concentration was determined by OD_{280}, and purity was determined using SDS-PAGE.

A significant contamination from bovine IgG (present in the FBS) was noted. Even though the domains 1-3 fusion protein was predicted to be smaller than the domains 4-8 fusion protein, both migrated at approximately 90 kD. One possible explanation for the observation is that the smaller domains 1-3 fusion protein may be more heavily glycosylated than the larger domains 4-8 fusion protein.

In addition to use of the purified proteins for monoclonal antibody production, described below, the proteins will also be used in adhesion assays to identify ICAM-4 ligands.

EXAMPLE 14

Monoclonal Antibody Production

The purified protein described in Example 13 was utilized to generate monoclonal antibodies using an immunization protocol as described in Example 6.

The spleen from mouse #2250 (immunized with HuICAM-4 D1-3/IgG1) was used for fusion 172 and the spleen from mouse #2272 (immunized with HuICAM-4 D4-8/IgG1) was used for fusion 173. The fusion protocol utilized was as described in Example 6. Fusion plates were screened by ELISA (essentially as described in Example 6) using each HuICAM-4/IgG1 fusion protein. Fusion well supernatants that recognized the immunogen protein, and no other, were considered for cloning. Immunocytochemistry on human hippocampus sections was used as a secondary screen.

One primary clone from each fusion was positive by immunocytochemistry and was cloned. One of the two clones failed to grow upon cloning, leaving only one candidate to pursue, clone 173E which was derived from the HuICAM-4 D4-8/IgG1 immunized mouse. Hybridoma 173E was

deposited June 1, 1995 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, and assigned Accession Number HB11912.

5   From another fusion derived from a mouse immunized with a soluble ICAM-4 fragment corresponding to domains 1-3, six clones (179A, 179B, 179D, 179H, 179I, and 179K) were found to be specific for HuICAM4 domains 1 through 3 (D1-3). All six antibodies in the 179 series bound to the dendritic processes in the dentate gyrus, as well as the polymorphic and pyramidal cell layers. The monoclonal antibody 179A stained neuronal cell

10   bodies from these areas in addition to the dendritic processes. The hybridoma cell lines producing antibodies 179I and 179H were deposited on June 10, 1996 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville Maryland, 20852 and assigned Accession Numbers HB 12123 and HB 12124, respectively.

15   Additional fusions are similarly performed to generate other antibodies specifically immunoreactive with particular ICAM-4 regions.

EXAMPLE 15

Capture Assay Development

From another fusion
The six monoclonal antibodies from fusion 179 were tested in

20   various combinations for their ability to capture and detect soluble ICAM-4 in solution. The assay, as described below, was established in order to evaluate soluble ICAM-4 levels in human fluids in relation to normal and disease conditions.

Antibody 179I was coated on Immulon 4 (Dynatech) 96 well

25   plates at 3 μg/ml, 125 μl/well for two hours at 37°C. The antibody solution was removed by aspiration and the wells were blocked for 30 minutes at room temperature with 300 μl of blocking solution containing 5 % Teleostean gelatin in calcium-free, magnesium-free PBS (CMF-PBS). The blocking solution was removed by aspiration, a 100 μl of sample fluid diluted in Omni Diluent

(CMF-PBS, 1% gelatin, and 0.05% Tween 20) was added to each well, and the mixture incubated at 37°C for 30 minutes. The plates were washed three times with PBST (CMF-PBS, 0.05% Tween 20). Antibody 179H was biotinylated at 1.5 mg/ml using NHS-LC-Biotin (Pierce) following suggested

5   manufacturer's protocol, diluted 1:2000, and added to the wells (100 μl/well). The resulting mixture was incubated for 30 minutes at 37°C and the plates washed three times with PBST. Streptavidin-HRP (Pierce) was added (100 μl, 0.25 μg/ml) to each well and this mixture incubated at 37°C for 30 minutes. The plates were washed four times with PBST before addition of

10   100 μl of Tetramethylbenzidine (Sigma) (10 mg/ml stock in DMSO) diluted 1:100 in buffered substrate (13.6 g/L sodium acetate trihydrate, pH to 5.5 with 1 M citric acid, with 150 μl/L 30% hydrogen peroxide added just prior to developing). The reaction was allowed to develop for 30 minutes at room temperature in the dark, after which the reaction was stopped with addition of

15   50 μl/well 15% H_2SO_4. The absorbance was read at 450 nm.

Results indicated that the assay was capable of detecting soluble HuICAM-4 D1-3 recombinant protein at a concentration as low as 5-10 ng/ml with the linear portion of the curve being in the 10 - 100 ng/ml range. No cross-reactivity to HuICAM4 D4-8 was observed when this protein region was

20   tested at 1 and 10 μg/ml.

EXAMPLE 16

Assessment of Soluble ICAM-4 in Serum from Stroke Patients

In order to assess the role of ICAM-4 in neurologic diseases and conditions, serum from twenty-eight patients suffering from acute stroke

25   and twenty young healthy volunteers (not age matched) was assayed as described above for differences in serum concentration of soluble ICAM-4.

Results indicated that serum from the healthy volunteers had no detectable level of ICAM-4. Twenty out of twenty-eight acute stroke patients, however, had detectable levels of soluble ICAM-4. The signal from the

positive stroke patients corresponded to a range of 5-38 ng/ml of the standard (soluble ICAM-4 D1-3 recombinant protein).

EXAMPLE 17

ICAM-4 mRNA Levels in Hippocampus in a Rat Model of Epilepsy

5   Levels of rat ICAM-4 mRNA expressed were assessed in hippocampus of rats treated in a manner to create a kindling epileptogenesis animal model [Lothman, *et al.*, *Brain Res.* 360:83-91 (1985)]. In the model, the rat hippocampus is stimulated with a series of subconvulsive electric shocks through an electrode implanted in the region of the brain which

10   gradually elicits severe behavioral seizures. The kindling process involves twelve stimulations per day administered every other day for eight days. Once fully kindled, a single stimulus can elicit behavioral seizures and histologic changes that are similar to human epilepsy. Fully kindled rats received two stimulations per day over a two week period and animals were

15   sacrificed 24 hours after the last stimulation. The hippocampus was removed and dissected for RNA preparation.

          Total RNA was prepared from each sample using the guanidinium/phenol/chloroform extraction procedure [Chomezynski and Sacchi, *Anal. Biochem.* 162:156-159 (1987)]. RNA was separated on denaturing

20   formaldehyde agarose gels, transferred to nylon membranes, and hybridized with radiolabelled rat ICAM-4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific DNA probes. GAPDH is a basally expressed gene that is commonly used as a control to detect lane to lane variation in the amount of RNA loaded on a gel. Fluctuations in the ratio of the ICAM-4/GAPDH are

25   interpreted as changes in the level of ICAM-4 expression. Hybridizing bands for ICAM-4 and GAPDH were quantitated with a phosphorimager and a ratio of ICAM-4/GAPDH determined.

          The ratio of ICAM-4/GAPDH was significantly higher in the control animals that were not kindled (n=5) compared to the kindled test

group (n=5), suggesting that ICAM-4 was down regulated as a consequence of the kindling process. It should be noted, however, that the control group did not undergo any sham treatment so the possibility exists that ICAM-4 mRNA levels were modulated in response to the surgical treatment associated with kindling.

EXAMPLE 18

Serum ICAM-4 Concentration as a
Marker for Neurodegenerative Disorders

Circulating serum concentrations of ICAM-4 were assessed as a possible indicator for various neurodegenerative disorders. Serum and/or plasma samples from anonymous donors were assayed as described in Example 16 above and compared to samples drawn from control donors with no previous history of neurological disorders.

Control Donors

In order to establish a baseline average for circulating ICAM-4 in normal healthy individuals, serum samples from 100 donors were examined. The results showed that twelve individuals (12%) had circulating levels of ICAM-4 greater than 10 ng/ml. Of these twelve, the ICAM-4 concentration in five samples averaged 10-20 ng/ml, three samples showed an average ICAM-4 concentration of 20-100 ng/ml, two samples showed ICAM-4 levels between 100-500 ng/ml, and two samples contained ICAM-4 at a concentration in excess of 500 ng/ml.

Samples were taken at the same time from both donors with very high levels at varying timepoints over an eight month period to assess the stability of the observations over time. It was observed that over a period of months, the readings did fluctuate. No medical information was available on these donors, making correlations with the ICAM-4 levels and the physical well-being of the donors not possible. When both serum and plasma samples

were prepared from the same individual, no difference was observed in the level of ICAM-4 present.

This observation indicated that an assay for soluble ICAM-4 would be versatile in its use of either serum or plasma. In addition, the results indicated that ICAM-4 is very stable in blood, suggesting that an elevated level of ICAM-4 as a result of some pathological state probably would not be transient. Finally, because of the apparent stability of ICAM-4 in a blood environment, assays for soluble ICAM-4 can utilize blood bank samples thus reducing the need for fresh blood with each assay.

In order to determine if the methods of collection and/or storage affected the observations, the stability of ICAM-4 serum was assessed by treating samples from the one individual with the highest level of circulating ICAM-4 in a variety of ways followed by a measurement of the levels of ICAM-4. Neither incubation at 37°C for 24 hours nor from one to three freeze/thaw cycles altered the level of detectable ICAM-4 in the serum.

Donors with Epilepsy

The serum concentration of ICAM-4 in samples from twenty patients with Temporal Lobe Epilepsy (TLE) was measured and compared to serum samples from control group patients that had experienced Grand Mal Seizures (38 different patients), Syncope (8 patients) or were normal healthy donors (20 individuals). The assay method described in Example 15 was again employed and the results expressed as ng/ml relative to the internal standard used for the assay, soluble HuICAM-4 D1-3 recombinant protein (described in Example 13).

Serum from all 20 patients with TLE had measurable levels of ICAM-4 with an average of approximately 140 ng/ml. In serum samples from all 3 control groups, including the Grand Mal Seizure group, ICAM-4 concentration averaged below 10 ng/ml. These observations suggest that an individual's ICAM-4 serum level may represent a biochemical marker which

can distinguish between focused seizures, like those experienced in TLE, and more generalized Grand Mal Seizures.

Donors with AIDS

Serum concentration of ICAM-4 in the sera from a limited number of AIDS patients was also examined. The patients were grouped according to CD4 counts and the presence of any signs of dementia. A first group comprised sixteen early stage, asymptomatic patients with CD4 counts greater than 500 were tested. A second group comprised seven later stage patients with CD4 counts less than 300; signs of dementia were not determined for this group. The last group comprised nine late stage AIDS patients, each showing signs of dementia.

The results showed that serum samples from four of the sixteen (25%) early stage, asymptomatic patients had detectable levels of soluble ICAM-4; three of the four samples had an ICAM-4 concentration in excess of 500 ng/ml. Four of the seven (57%) serum samples from later stage patients were also positive for ICAM-4, with two of the four having ICAM-4 concentrations in excess of 500 ng/ml. Samples from the late stage patients showing signs of dementia had no detectable levels of ICAM-4. The results of this preliminary study suggest that ICAM-4 may be an early marker of the neurodegeneration associated with AIDS dementia.

Donors with Other Neurodegenerative Diseases

The results from the study of serum from epilepsy and AIDS donors suggest that ICAM-4 levels in the blood may reflect damage to the neurons that normally express it. There are a number of other neurologic diseases that might also show, as part of their etiology, damage to specific ICAM-4 expressing neurons that could result in changes in the serum concentration of ICAM-4 in the periphery.

For example, Alzheimer's disease is associated with extensive neuronal damage in the regions of the telencephalon where ICAM-4 is expressed. Assessment of ICAM-4 levels in serum from patients with the Early-onset Familial forms of the disease, as well as patients with the sporadic form of the disease, may provide a marker for the various stages of the disease thereby permitting assessment of possible therapeutic interventions.

As another example, because other cortical dementias, such as Pick's disease, diffuse cortical Lewy body disease, and frontal lobe degeneracy, are sometimes mistaken for Alzheimer's, but may be distinguishable from each other and from Alzheimer's disease through serum ICAM-4 analysis. As another example, serum ICAM-4 concentration in patients suffering from a subcortical dementia, including Parkinson's disease, Huntington's disease, and progressive supranuclear, may be elevated as a result of common pathological indications of this class of disorders.

As another example, a number of the primary psychiatric disorders, such as depression, schizophrenia and psychosis, are characterized in part by degrees of neurodegeneration that might be associated with detectable levels of ICAM-4 in the blood.

As another example, elevated levels of ICAM-4 may be associated with a number of nongenetic dementias arising from infections, vasculitis, metabolic and nutritional disorders (*e.g.*, thyroid, vitamin B12 deficiency), vascular disorders (multiple infarct, lacunar state, Binswanger's disease), toxic encephalopathies (*e.g.*, exposure to carbon monoxide, heavy metals or other industrial pollutants) and tumors.

EXAMPLE 19
Cloning and Analysis of Human ICAM-4 Upstream Regulatory DNA

ICAM-4 gene expression is spatially and temporally regulated, with expression limited to the most anterior or ventral region of the brain, the telencephalon. In an attempt to identify gene sequences responsible for the

restricted transcriptional regulation of ICAM-4, the nucleotide region 5´ to human ICAM-4 coding sequences was examined.

A 2607 base pair *Bam*HI/*Pst*I fragment derived from a 7.0 kb genomic *Bam*HI fragment (described in Example 10) was sequenced and found to contain 1684 nucleotides upstream of the ATG start codon. The complete sequence for this upstream region is set out in SEQ ID NO: 33. With respect to the position of the ICAM-4 coding region, the "A" in ATG start codon (numbered in SEQ ID NO: 33 as nucleotides 1685-1687) is designated the $+1$ nucleotide and the nucleotide immediately 5´ to the A^{+1} nucleotide is designated -1. Thus the entire sequence is shown as extending from nucleotide -1684 to nucleotide $+3$, corresponding to numbering in the Sequence Listing nucleotide 1 to nucleotide 1687.

Based on the genomic HuICAM-4 sequence, oligonucleotides were synthesized and used in PCR to generate DNA molecules of various lengths within the upstream regulatory region. Each oligonucleotide set out in Table 3 contained a spacer region (shown in italics) approximately 6-10 bp to allow enzymatic digestion of the PCR product, an *Nhe*I or *Hind*III restriction site (shown in bold), and a specific hybridization primer sequence (underlined). The oligonucleotide names contain numbers that designate its location within the upstream regulatory region. In the PCR amplifications, oligonucleotides were paired as shown in Table 4 to generate DNA fragments containing specific regions of the upstream regulatory region.

The restriction sites and spacer region generated within each oligonucleotide allowed for enzymatic digestion and subsequent directional cloning of individual PCR products into the pGL3 Basic Vector (Promega, Madison, WI) which contains a luciferase reporter gene immediately downstream of a multiple cloning site (MCS). Promoter activity cloned into the MCS region of the vector drives expression of the luciferase reporter gene in transfected cell lines, and light production from expressed luciferase can be measured as an indicator of promoter activity. The pGL3 Basic Vector has

TABLE 3

PCR Primers Used to Amplify HuICAM-4 Upstream Regions

HI4-19(AS)	*CAGAAC*TAAGCTT<u>ACAGGAGGCGAGGAGAGCGCGAG</u>	
		(SEQ ID NO: 34)
5	HI4-114	*CAACAAT*GCTAGC<u>CAAGCGCAACTCTGTCTC</u>
		(SEQ ID NO: 35)
	HI4-149	*CAACAAT*GCTAGC<u>CTTGGAAACCAAGTTACC</u>
		(SEQ ID NO: 36)
10	HI4-206	*CAACAAT*GCTAGC<u>AGGAGCTTAGCGCACGCTCG</u>
		(SEQ ID NO: 37)
	HI4-270	*CAACAAT*GCTAGC<u>CATGCCGGCCTCCACGTAG</u>
		(SEQ ID NO: 38)
	HI4-408	*CAACAAT*GCTAGC<u>GTCCAGCTTATTATCATG</u>
		(SEQ ID NO: 39)
15	HI4-480	*CAACAAT*GCTAGC<u>CTTAGTCCCCAAATGTATC</u>
		(SEQ ID NO: 40)
	HI4-560	*CAACAAT*GCTAGC<u>GGAGAAGGATCAGTGAG</u>
		(SEQ ID NO: 41)
20	HI4-817	*CAACAAT*GCTAGC<u>CTCCACCCACCGAGCAGAAG</u>
		(SEQ ID NO: 42)

no promoter and therefore served as the negative control, while a pGL3 vector containing an SV40 promoter served as a positive control. The sequence of each expression construct was verified by restriction analysis and DNA sequencing.

25   Plasmids containing each of the amplified sequences described in Table 4 were transfected into mammalian cells using a Transfection MBS Mammalian Transfection Kit (Stratagene, La Jolla, CA) according to manufacturer's suggested protocol. Each plasmid was introduced into two different cell lines, COS 7 and NT2 Precursor Cells (Ntera2/D1 from

Stratagene). COS 7 cells are a commonly used simian fibroblast-like cell line transformed with SV40 making them well suited for driving expression of a gene under control of the SV40 promoter in cells transfected with the positive control pGL3 Promoter Vector. NT2 precursor cells are a committed neuronal precursor cell line, and while they do not express ICAM-4, they may be more representative of a cell type that does express ICAM-4.

TABLE 4

Primers Paired and Regions Amplified

Oligonucleotide Pairs	Corresponding Upstream Regulatory Region
HI4-19 (AS) with HI4-114	$-19 \rightarrow -114$
HI4-19 (AS) with HI4-149	$-19 \rightarrow -149$
HI4-19 (AS) with HI4-206	$-19 \rightarrow -206$
HI4-19 (AS) with HI4-270	$-19 \rightarrow -270$
HI4-19 (AS) with HI4-408	$-19 \rightarrow -408$
HI4-19 (AS) with HI4-480	$-19 \rightarrow -480$
HI4-19 (AS) with HI4-560	$-19 \rightarrow -560$
HI4-19 (AS) with HI4-817	$-19 \rightarrow -817$

Each well of a 6 well flat bottom tissue culture plate (Falcon) was seeded with 2.5×10^5 cells. Transfections of COS 7 and NT2 cells were done side by side in duplicate using 5 μg of plasmid DNA for each well. The cells were cultured at 37°C for 48 hours, lysed and assayed for luciferase activity with a Luciferase Assay System (Promega).

Results of the experiment, summarized in Table 5, indicate a high level of promoter activity contained within the -408 through -19 and -480 through -19 regions of the upstream regulatory region of ICAM-4 in NT2 cells. Because NT2 cells are of neuronal origin, they may express certain transcription factors recognizing the ICAM-4 promoter that are not found in other cell types. The highest level of promoter activity in COS cell

transfectants was obtained with the plasmid containing nucleotides -560 through -19. While the positive control pGL3 Promoter Vector worked well in COS cells, it showed very low promoter activity in NT2 cells, thus illustrating a cell type specific preference for certain promoter sequences.

TABLE 5

Promoter Activity of 5´ ICAM-4 Regions

Upstream Region	Luminescence	
	COS	NT2
-114 through -19	0.003	0.376
-149 through -19	0.008	0.628
-206 through -19	0.443	0.622
-270 through -19	0.056	1.140
-408 through -19	0.401	7.970
-480 through -19	0.274	4.630
-560 through -19	3.227	1.232
-817 through -19	0.035	4.453
pGL3 Promoter Vector	29.070	0.063
pGL3 Basic Vector	0.008	0.014

Since neither COS 7 or NT2 cells normally express ICAM-4, the same experiment will be repeated using primary cultured rat hippocampal neurons which do express ICAM-4 and necessarily express transcriptional machinery required for ICAM-4 promoter activity. By transfecting the individual promoter constructs described herein, as well as others, into the more natural environment, it may be possible to identify more precisely which nucleotides in the upstream regulatory region are responsible for tight regulation of the ICAM-4 gene in the brain.

The foregoing illustrative examples relate to presently preferred embodiments of the invention and numerous modifications and variations thereof will be expected to occur to those skilled in the art. Thus only such limitations as appear in the appended claims should be placed upon the scope of the present invention.